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Title of the Invention

Co-administration of a Thrombolytic and an anti-CD18 Antibody

10 Related Applications

This application is a continuation-in-part of U.S. Serial No. 08/788,800 filed 22 January 1997, which claims priority to U.S. Serial No. 08/689,982 filed 23 January 1996, and claims priority to provisional application U.S. Serial No. 60/093,038 filed 23 January 1996, the contents of which are incorporated herein by reference in their entirety.

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Background of the Invention

The invention is related to a method of co-administering a thrombolytic compound and an anti-CD18 antibody.

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Of the nearly 750,000 people who suffer an acute myocardial infarction (AMI) in the United States each year, approximately 250,000 will receive some form of reperfusion therapy. While reperfusion therapy with either thrombolysis or primary percutaneous transluminal coronary angioplasty (PTCA) has been shown to improve survival, animal models suggest that reperfusion therapy may also be associated with what has been described as "lethal reperfusion injury," defined as myocardial cell death that occurs secondary to the reperfusion and not from the preceding ischemia.

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It is unclear whether reperfusion injury occurs in humans. In randomized-controlled trials, thrombolytic therapy has been shown to successfully establish epicardial flow in the infarct related artery (IRA) in ~80% of subjects (GUSTO, 1993). However, recent studies have shown that patients with sluggish flow in the IRA (Thrombolysis In Myocardial Infarction or TIMI grade 2 flow) have a much worse prognosis than those patients with brisk flow (TIMI grade 3 flow) (Anderson et al., 1996). In fact, the prognosis of patients with TIMI grade 2 flow is similar to patients with an occluded IRA

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(TIMI grade 0-1 flow) (Anderson et al., 1996). Laster et al., (1996) evaluated 180 patients who underwent primary PTCA to determine the association between angiographic flow and myocardial salvage and final infarct size. Using predischARGE
5 perfusion imaging with Technetium-99m (Tc-99m) Sestamibi, they found that infarct size was significantly smaller in patients with TIMI grade 3 flow than in those with TIMI grade 2 flow (\pm SD) ($15\pm 16\%$ vs. $29\pm 21\%$ of left ventricular mass, $p=0.007$). Furthermore, the myocardial salvage index was $55\pm 41\%$ of the area at risk in the TIMI 3 group and $27\pm 38\%$ of the area at risk in the TIMI 2 group ($p=0.04$). Thrombolysis is not, therefore, completely effective when used alone for those patients with resulting TIMI
10 grade 2 flow or lower.

In a further attempt to understand the mechanism of reduced flow following thrombolysis, Gibson et al. (1997) examined patients who received thrombolytic therapy and underwent rescue PTCA and intracoronary stent placement. They concluded that residual luminal stenosis is not responsible for the development of TIMI grade 2 flow.
15 Ito et al. (1992, 1996a, 1997) examined whether TIMI grade 2 flow might represent a form of no reflow. Using myocardial contrast echocardiography, they demonstrated that patients with TIMI grade 2 flow had no evidence of perfusion at the cellular level. This observation is consistent with previous work done by Schofer et al. (1985), who also demonstrated using Technetium-labeled albumin that no reflow exists in some patients
20 with AMI following reperfusion therapy. A need continues to exist for methods of improving the clinical outcome of patients having blocked arteries in order to reduce the number of patients demonstrating a TIMI grade 2 flow or lower.

A family of adhesion glycoproteins present on leukocytes is called the integrin family. This integrin family includes LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and
25 p150,95 (CD11c/CD18). A further member of this family CD11d/CD18 has been reported. Danilenko *et al.*, (1995). Each of these heterodimers has a unique alpha-chain (CD11a, b, c or d) and an invariant beta-chain (CD18). CD18 integrins located on leukocytes bind to intercellular adhesion molecule-1 (ICAM-1) which is expressed on
30 transendothelial migration.

The mechanism by which lethal reperfusion injury occurs in animal models of ischemia/reperfusion is unknown. However, there is emerging evidence to suggest that neutrophils may play a role. At the onset of reperfusion, neutrophils become activated and adhere to the previously ischemic endothelium. Adhesion occurs when the CD11b/CD18 receptor on the neutrophil binds to the intracellular adhesion molecule (ICAM) receptor on the endothelial cell. The neutrophils then migrate by diapedesis into the extravascular space, where they release azurophilic granules, increase their oxygen uptake, and cause free radical formation (Diacovo et al., 1996). One manifestation of reperfusion injury is the so-called "no-reflow" phenomenon (Kloner et al., 1974). No reflow occurs when recanalization of a large epicardial coronary artery fails to result in perfusion to the microvascular bed. The no-reflow phenomenon appears to be caused by both microvascular endothelial dysfunction (Ma et al., 1991b) and mechanical obstruction due to neutrophil plugging (Kloner et al., 1974).

Investigators have attempted to limit reperfusion injury by inhibiting neutrophil-endothelial cell binding during reperfusion with the administration of various antibodies directed against the CD18 receptor. These nonclinical studies demonstrate that anti-CD18 antibodies reduce reperfusion injury and lead to an improvement in coronary artery blood flow and a reduction in myocardial infarct (MI) size (Hernandez et al., 1987; Engler et al., 1986; Aversano et al., 1995; Lefer et al., 1995; Ma et al., 1991a).

The safety and efficacy of delayed therapy with tPA has been investigated (Gross, et al., (1995, Neurosurgery, 36:1172-1177). The effect of anti-ICAM-1 antibodies in a rabbit embolic stroke model followed by thrombolysis with tPA has also been examined (Bowes et al., 1993, Exp. Neurol., 119:215-219). Although tPA (30 min postischemia) and anti-ICAM-1 antibody (5 min postischemia) each separately improved the neurological outcome relative to controls, the combination of the two compounds at the same times was no more effective than either compound alone. When thrombolysis was delayed 3 hr following embolism, neither tPA nor the combination reduced neurological damage. Experiments in rabbits have also shown that tPA (30 min postischemia) and an anti-CD18 antibody (5 min postischemia) each separately improved neurological outcome, although the combination of the two compounds at the same times was no more effective than either compound alone (Bowes et al., (1995)). The combination of anti-

ICAM-1 antibody (15 min postischemia) and tPA (2 hr postischemia) extended the postischemia duration at which the tPA remained effective. That is, the combination was effective in extending the therapeutic window of tPA outside the effective therapeutic window of the tPA when administered alone in a rabbit. This effect has also been seen in rats. Ruilan et al., (1998). In this study, administration of tPA and an anti-CD18 antibody to rats four hr postischemia extended the therapeutic window for thrombolysis in rats.

No studies have been reported which are directed to studies of blood flow, infarct size or clinical outcomes in human patients treated with both thrombolytics and anti-CD18 antibodies. Further, prior art studies suggest only that the therapeutic window for thrombolysis may be extended in the animal models studied. That is, these studies indicate only that the administration of a thrombolytic and an anti-CD18 antibody may allow the thrombolytic to function effectively, i.e. to dissolve a clot, for a period of time which is longer than the effective time period in the absence of the antibody. These studies do not suggest or show that blood flow can be increased in an artery having reduced flow following thrombolysis, that infarct size can be reduced or that clinical outcomes can be improved in patients, particularly in human patients, treated with a thrombolytic and an antibody, in particular during the normal effective therapeutic window of the thrombolytic. Accordingly, there continues to be a need for a method of improving the clinical outcomes of patients, increasing blood flow in arteries following thrombolysis, decreasing infarct size and/or reducing the number of patients demonstrating reduced blood flow after treatment with a thrombolytic.

Summary of the Invention

Accordingly, one object of the present invention is to provide a general method of improving clinical outcomes , for example, by increasing blood flow in an artery at least partially blocked and containing a thrombus (for example, in human patients who have suffered an AMI or stroke) and/or by reducing infarct size (for example, in human patients with AMI or stroke). Surprisingly, it has been discovered that co-administration of a thrombolytic compound and an anti-CD18 antibody is more effective at improving clinical outcomes than tPA alone. This is in contrast to the results of prior experiments

which indicated that a combination therapy using both tPa and an anti-CD18 antibody was no more effective than either compound used alone under the conditions tested.

The invention provides a general method which involves co-administering a thrombolytic compound and an anti-CD18 antibody.

5 The invention also provides a method for increasing blood flow in an infarct related artery in a mammal (e.g. AMI in a mammal with a blocked coronary artery or focal ischemic stroke caused by obstruction of a cerebral artery) by administering amounts of an anti-CD18 antibody and a thrombolytic agent to the mammal, for example a human patient, which are effective for increasing arterial blood flow in the mammal.

10 The invention further provides a method of treating a human patient at risk of having Thrombolysis In Myocardial Infarction (TIMI) grade 2 or less blood flow in an infarct related artery (IRA) at least partially occluded by a thrombus or embolus by co-administering effective amounts of a thrombolytic compound and an anti-CD18 antibody to a patient in need thereof.

15 The invention also provides a method for treating a group of patients having AMI which reduces the number or percentage of treated patients demonstrating a TIMI grade 2 or lower score after the treatment, relative to the number of patients treated with a thrombolytic alone or with a combination of a thrombolytic and angioplasty, by administering effective amounts of an anti-CD18 antibody and a thrombolytic agent to
20 the patient.

 The invention further provides a method of treating a human AMI patient who has been treated with a thrombolytic compound which dissolves or removes a thrombus or embolus from an infarct related artery (IRA) at least partially occluded by the thrombus or embolus, by administering an effective amount of an anti-CD18 antibody to the patient
25 in need thereof during the effective therapeutic window of the thrombolytic compound administered alone.

 The preferred mode of administration of the compounds of the invention in each of these embodiments is by bolus intravenous dosage. Single or multiple dosages may be given. Alternatively, or in addition, the antibody and thrombolytic agent may be
30 administered via continuous infusion.

Brief Description of the Figures

Fig. 1 is a bar graph depicting brain infarct size (% hemisphere infarcted) in embolized rabbits following treatment with MHM23 (anti-CD18) and t-PA (n=5); MHM23 alone (n=5); t-PA alone (n=10) or saline solution control (n=10) as described in Example 1 (mean +/- standard error of the mean).

Fig. 2 depicts regional cerebral blood flow (CBF; cc/100gm/min) over time in embolized rabbits following treatment with MHM23 (anti-CD18) and t-PA (n=5); MHM23 alone (n=5); t-PA alone (n=10) or saline solution control (n=10) as described in Example 1. MHM23 or saline solution control was administered 1 hour following embolization. t-PA or saline solution control was administered by continuous infusion over hours 3-5 following embolization (mean +/- standard error of the mean).

Fig. 3 illustrates intracranial pressure (ICP; mm Hg) in embolized rabbits following treatment with MHM23 (anti-CD18) and t-PA (n=5); MHM23 alone (n=5); t-PA alone (n=10) or saline solution control (n=10) as described in Example 1. MHM23 or saline solution control was administered 1 hour following embolization. t-PA or saline solution control was administered by continuous infusion over hours 3-5 following embolization (mean +/- standard error of the mean).

Fig. 4 shows a saturation vs. time curve for rhuMab for a single dose in humans at 0.5 mg/kg and 2.0 mg/kg.

Fig. 5 shows serum levels after IV bolus administration of two doses of rhuMab CD18 at 0 and 12 hrs in Study PDL 2000P.

Fig. 6 shows serum levels after IV bolus administration of a single dose of rhuMab CD18 in Study PDL 2001P.

Fig. 7 shows serum levels after IV bolus administration of two doses of rhuMab CD18 at 0 and 12 hrs in Study PDL 2001P.

Definitions

"Acute myocardial infarction" (AMI) is defined as immediate or sudden (not chronic) infarction of the heart muscle, that is, an insufficiency of arterial blood as a result of occlusion of a coronary artery due to at least partial blockage of the artery by an embolus or thrombus. As used herein, "thrombus" and "embolus" refer to a blood clot

within a blood vessel. "At least partial" blockage of an artery means that the artery contains an embolus or thrombus which reduces the cross sectional area of the artery. The reduction may be as little as 10% of the cross sectional area of artery or may completely block the artery 100%; frequently the reduction in area will be in the range of about 50% to about 80% of the artery. As used herein, the term AMI does not include non-thrombus surgical blockage or stricture of an artery with a device or method other than an embolus or thrombus, for example using a microaneurysm clip, a catheter balloon or nylon filament, introducing polymer microspheres into an artery, etc.

As used herein, "a patient at risk of having Thrombolysis In Myocardial Infarction (TIMI) grade 2 or less blood flow in an infarct related artery (IRA)" means a patient who has or has had an AMI and who is or will be treated with a thrombolytic compound. As described above, a portion (about 25%) of AMI patients who are treated with a thrombolytic compound do not respond with a TIMI grade 3 flow, but rather demonstrate a TIMI blood flow which is grade 2 or less even after treatment with the thrombolytic compound. Any patient who has had an AMI and is or will be treated with a thrombolytic compound is, therefore, "at risk of having TIMI grade 2 or less blood flow in an infarct related artery (IRA)." Patients "at risk" may also be treated with angioplasty, so long as they receive at least one dose of a thrombolytic compound according to the invention.

"Angioplasty" or "percutaneous transluminal angioplasty" or "percutaneous transluminal coronary angioplasty" (PTCA)" is an operation for enlarging a narrowed arterial lumen by peripheral introduction of a balloon-tip catheter and dilating the lumen on withdrawal of the inflated catheter tip.

"Co-administration" or "co-administering" as used herein means the administration of an anti-CD18 antibody during the effective therapeutic window of the thrombolytic compound administered alone. The antibody may be administered before, concurrent with or after the thrombolytic compound. The administration of the antibody is preferably started from about 1 hr before up to immediately (1-15 min) before, more preferably concurrently with, the start of administration of the thrombolytic. Co-administration also encompasses administration of the antibody after the start of

administration of the thrombolytic, for example about 15-30 min after and up to about 3 hr after.

The "effective therapeutic window" of a thrombolytic compound administered alone means the time period or time window following an infarct caused by blockage of an artery during which a thrombolytic compound, when administered alone, is effective in reestablishing patency of blood flow in the artery relative to a control not receiving the thrombolytic compound. The effective therapeutic window is species dependent for any particular thrombolytic compound, but can be readily determined by standard tests evaluating the efficacy of the thrombolytic treatment versus controls. For tPA administered in humans, the effective therapeutic window is 0-12 hrs following an infarct.

"Focal ischemic stroke" is defined herein as damage to the brain caused by interruption of the blood supply to a region thereof. The focal ischemic stroke of interest is generally caused by obstruction of any one or more of the "main cerebral arteries" (*e.g.* middle cerebral artery, anterior cerebral artery, posterior cerebral artery, internal carotid artery, vertebral artery or basilar artery), as opposed to secondary arteries or arterioles. The "arterial obstruction" is generally a single embolus or thrombus. Hence, focal ischemic stroke as defined herein is distinguished from the cerebral embolism stroke model of Bowes et al.(1995) in which a plurality of clot particles occlude secondary arteries or arterioles.

By "increasing blood flow" is meant the act of improving clinical outcome by inducing a statistically or physiologically significant increase in arterial blood flow in a treated mammal relative to an untreated mammal as determined using techniques which are well known in the art, such as vascular imaging, for example. Preferably arterial blood flow, as determined at about 90 minutes after administration of the thrombolytic compound, is increased by at least 30% and preferably at least 50% relative to an mammal treated with the thrombolytic alone, and preferably to a TIMI grade 3 flow. When a group of AMI patients is treated, the number of treated patients demonstrating a TIMI grade 2 flow or lower after treatment according to the invention is preferably decreased by at least 30%, preferably at least 50%, relative to a group of AMI patients receiving the thrombolytic compound, but not the antibody.

An "infarct" is an area of necrosis in a tissue or organ, for example heart or brain, resulting from obstruction of the local circulation by a thrombus or embolus. Infarct size can be measured by known methods.

5 An "infarct related artery" is an artery which when at least partially blocked by a thrombus or embolus gives rise to an infarct in a tissue or organ, for example heart or brain.

The term "anti-CD18 antibody" when used herein refers to an antibody which binds to CD18 (preferably human CD18) and inhibits or substantially reduces a biological activity of CD18. Normally, the antibody will block (partially or completely) the ability of a cell (*e.g.* a neutrophil) expressing the CD18 subunit at its cell surface to bind to endothelium.

Examples of anti-CD18 antibodies include MHM23 (Hildreth *et al.* (1983)); M18/2 (IgG_{2a}; Sanches-Madrid *et al.*, (1983)); H52 (American Type Culture Collection (ATCC) Deposit HB 10160); Mas191c and IOT18 (Vermot Desroches *et al.*, (1991)); and NA-8 (WO 94/12214). The preferred antibody is one which binds to the CD18 epitope to which either MHM23 or H52 binds. Preferably the antibody has a high affinity for the CD18 polypeptide. In preferred embodiments, the antibody has an affinity for the CD18 antigen of about 4nM or less. Preferably, the affinity is about 3nM or less, and most preferably 1nM or less. In certain embodiments, the antibody may bind to a region in the extracellular domain of CD18 which associates with CD11b and the antibody may also dissociate alpha and beta chains (*e.g.* the antibody may dissociate the CD11b and CD18 complex as is the case for the MHM23 antibody).

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies, antibody compositions with polyepitopic specificity, bispecific antibodies, diabodies, and single-chain molecules, as well as antibody fragments (*e.g.*, Fab, F(ab')₂, and Fv), so long as they antagonize the biological activity of CD18.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional

(polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.*, (1975), or may be made by recombinant DNA methods (see, *e.g.*, U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.*, (1991) and Marks *et al.*, (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; Morrison *et al.*, (1984)).

"Humanized" forms of non-human (*e.g.*, murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are found

neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al.*, (1986); Reichmann *et al.*, (1988); and Presta, (1992). The humanized antibody includes a PrimatizedTM antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H - V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger *et al.* (1993).

As used herein, the term "salvage receptor binding epitope" refers to an epitope of the Fc region of an IgG molecule (*e.g.*, IgG₁, IgG₂, IgG₃, and IgG₄) that is responsible for increasing the *in vivo* serum half-life of the IgG molecule.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. This application is mostly concerned with

treating those individuals who have been diagnosed as having suffered a blocked artery, such as AMI or acute ischemic stroke.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, *etc.* Preferably, the mammal is human.

A "thrombolytic compound or agent" is a molecule which breaks up or dissolves a thrombus. Exemplary thrombolytic agents include streptokinase, acylated plasminogen-streptokinase activator complex (APSAC), urokinase, single-chain urokinase-plasminogen activator (scu-PA), thrombinlike enzymes from snake venoms such as ancrod (Bell, W. "Defibrinogenating enzymes" In Colman *et al.*, (1987)), tissue plasminogen activator (t-PA) and biologically active variants of each of the above. The preferred thrombolytic agent is t-PA.

In the context of the present invention, the terms "tissue plasminogen activator" and "t-PA" are used interchangeably and denote extrinsic (tissue type) plasminogen activator having at least two functional domains consisting of a protease domain that is capable of converting plasminogen to plasmin and an N-terminal region believed to be responsible for fibrin binding. These terms therefore include polypeptides containing these functional domains as part of the overall amino acid sequence, irrespective of their source and method of preparation (*e.g.* these terms cover vampire bat t-PAs as disclosed in EP 352,119). The terms "human tissue plasminogen activator" and "human t-PA" are used interchangeably and denote wild-type human tissue plasminogen activator and functional derivatives thereof. Examples of t-PA functional derivatives include those molecules with extended half-life and improved fibrin specificity as disclosed in WO 93/24635; N-terminally truncated t-PA variants (see EP 382,174); and C84S t-PA described in Suzuki *et al.* (1993), for example. See also U.S. 5,869,314; 5,728,565 and 5,728,566.

Detailed Description of the Preferred Embodiments

In patients who are treated with a thrombolytic compound, the thrombolytic compound generally dissolves or removes the thrombus or embolus in the infarct related artery (IRA) and patency or blood flow through the IRA is reestablished. Nevertheless,

for about 25% of the patients, the patency status of the IRA does not indicate whether a patient will have a good clinical outcome. That is, a portion of patients treated with a thrombolytic alone or a combination of a thrombolytic and angioplasty still have a poor clinical outcome, sometimes as poor as if the patient had not been treated. The present invention provides a treatment which improves the overall clinical outcome of patients treated with a thrombolytic by administering an anti-CD18 antibody before, during or after administration of the thrombolytic compound.

In the method of the invention, clinical outcomes are improved by coadministration of a thrombolytic compound and an anti-CD18 antibody to a patient in need of such treatment. It is contemplated that the anti-CD18 antibody and thrombolytic compounds ("compounds of the invention") of the present invention will be administered to a patient as soon as possible once the condition of a blocked artery has been diagnosed, for example, through the use of a conventional angiogram or symptom recognition or is suggested by focal deficit on physical examination. Examination and, optionally, imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) (including diffusion weighted imaging (DWI) and perfusion imaging (PI)); vascular imaging (*e.g.*, duplex scanning and transcranial Doppler ultrasound and laser Doppler); angiography (*e.g.*, computerized digital subtraction angiography (DSA) and MR angiography) as well as other invasive or non-invasive techniques are available for the diagnosis.

Preferably, the anti-CD18 antibody will be administered at least once or continuously with the thrombolytic agent ("co-administration"). In certain embodiments, the anti-CD18 antibody is administered as a single dose to the patient at a time from about 15-30 minutes prior to administration of the thrombolytic agent to a time about 3 hrs after administration of the thrombolytic agent. Preferably, the antibody is administered before the thrombolytic, however, the antibody may be administered concurrent with the thrombolytic or after the thrombolytic, more preferably within about 1-5 minutes of the thrombolytic initial dose. The dose of the antibody will generally range from about from about 100µg/kg to about 20mg/kg, and preferably from about 500µg/kg to about 5 mg/kg, for example 0.5-2.0mg/kg, and most preferably from about 1mg/kg to about 3mg/kg of the anti-CD18 antibody as an initial candidate dosage for

administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays elaborated herein.

5 The preferred antibody for use in the above method is humanized H52 antibody (huH52), especially the huH52 F(ab')₂ antibody fragment. In other embodiments, the full length IgG₂ huH52 antibody may be the molecule of choice. See WO 94/04679 and WO 96/32478.

10 Various other antibodies which bind to CD18 are available in the art. See U.S. Patent No. 5,817,515 and WO 94/02175. Furthermore, a description follows as to the production of anti-CD18 antibodies for use in the treatment of blocked arteries as defined herein.

15 Polyclonal antibodies are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride,
20 SOCl₂, or R¹N=C=NR, where R and R¹ are different alkyl groups.

25 Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining 1 mg or 1 µg of the peptide or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by
30 subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also
can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*, (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, (1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, (1984); Brodeur *et al.*, (1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson *et al.*, (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, (1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra *et al.*, (1993) and Plückthun, (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al.*, (1990). Clackson *et al.*, (1991) and Marks *et al.*, (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling

(Marks *et al.*, (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies. See also U.S. 5,750,373; 5,821,047; and 5,780,279.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567 (Morrison, *et al.*, (1984))), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

Chimeric or hybrid antibodies also may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide-exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, (1986); Riechmann *et al.*, (1988); Verhoeven *et al.*, (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567, wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR

residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims *et al.*, (1993); Chothia *et al.*, (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al.*, (1992); Presta *et al.*, (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in

chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, (1993); Jakobovits *et al.*, *Nature*, (1993); Bruggermann *et al.*, (1993). Human antibodies can also be derived from phage- display libraries (Hoogenboom *et al.*, *J. Mol. Biol.*, 227:381 (1991); Marks *et al.*, (1991)).

Bispecific antibodies (BsAbs) are antibodies that have binding specificities for at least two different epitopes. Exemplary BsAbs may bind to two different epitopes of the CD18 antigen. Such antibodies can be derived from full length antibodies or antibody fragments (e.g. F(ab')₂ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al.*, (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker *et al.*, (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal

ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It has been found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh *et al.*, (1986). Using such techniques, a bispecific molecule which combines a thrombolytic agent such as t-PA and an anti-CD18 antibody can be prepared for use in the treatment as defined herein.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. The following techniques can also be used for the production of bivalent antibody fragments which are not necessarily bispecific. For example, Fab' fragments recovered from *E. coli* can be chemically coupled *in vitro* to form bivalent antibodies. See, Shalaby *et al.*, (1992).

Various techniques for making and isolating bivalent antibody fragments directly from recombinant cell culture have also been described. For example, bivalent

heterodimers have been produced using leucine zippers. Kostelny *et al.*, (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers.

5 The "diabody" technology described by Hollinger *et al.*, (1993) has provided an alternative mechanism for making bispecific/bivalent antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the
10 complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific/bivalent antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber *et al.*, (1994).

In certain embodiments of the invention, it is desirable to use anti-CD18 antibodies engineered to have an enhanced half-life in the serum of a mammal treated
15 therewith. For example, this may be achieved by (i) incorporating a salvage receptor binding epitope of the Fc region of an IgG into the antibody so as to increase its circulatory half-life, but without disrupting its biological activity or (ii) covalently binding a nonproteinaceous polymer to the antibody. These exemplary techniques will be described briefly below:

20 Incorporation of a salvage receptor binding epitope into the antibody can take place by any means, such as by mutation of the appropriate region in the antibody of interest to mimic the Fc region or by incorporating the epitope into a peptide tag that is then fused to the antibody by DNA or peptide synthesis.

A systematic method for preparing such an antibody variant having an increased
25 *in vivo* half-life comprises several steps. The first involves identifying the sequence and conformation of a salvage receptor binding epitope of an Fc region of an IgG molecule. Once this epitope is identified, the sequence of the antibody of interest is modified to include the sequence and conformation of the identified binding epitope. After the sequence is mutated, the antibody variant is tested to see if it has a longer *in vivo* half-life
30 than that of the original antibody. If the variant does not have a longer *in vivo* half-life upon testing, its sequence is further altered to include the sequence and conformation of

the identified binding epitope. The altered antibody is tested for longer *in vivo* half-life, and this process is continued until a molecule is obtained that exhibits a longer *in vivo* half-life.

5 The salvage receptor binding epitope being thus incorporated into the antibody of interest is any suitable such epitope as defined above, and its nature will depend, *e.g.*, on the type of antibody being modified. The transfer is made such that the antibody of interest is still able to antagonize the biological activity of CD18.

10 An antibody contains an Ig domain or Ig-like domain and the salvage receptor binding epitope is preferably placed so that it is located within this Ig domain or Ig-like domain. More preferably, the epitope constitutes a region wherein any one or more amino acid residues from one or two loops of the Fc domain are transferred to an analogous position of the Ig domain or Ig-like domain of the antibody. Even more preferably, three or more residues from one or two loops of the Fc domain are transferred. Still more preferred, the epitope is taken from the CH2 domain of the Fc
15 region (*e.g.*, of an IgG) and transferred to the CH1, CH3, or V_H region, or more than one such region, of an Ig or to a Ig-like domain. Alternatively, the epitope is taken from the CH2 domain of the Fc region and transferred to the C_L region or V_L region, or both, of an Ig or to an Ig-like domain of the antagonist of interest.

20 Antibody-polymer conjugates can also be prepared. The nonproteinaceous polymer of choice for this purpose is ordinarily a hydrophilic synthetic polymer, *i.e.*, a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or *in vitro* methods are useful, as are polymers which are isolated from native sources. Hydrophilic polyvinyl polymers fall within the scope of this invention, *e.g.* polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are
25 polyalkylene ethers such as branched and unbranched polyethylene glycol (PEG); polyelkylenes such as polyoxyethylene, polyoxypropylene, and block copolymers of polyoxyethylene and polyoxypropylene (Pluronics); polymethacrylates; carbomers; branched or unbranched polysaccharides which comprise the saccharide monomers D-mannose, D- and L-galactose, fucose, fructose, D-xylose, L-arabinose, D-glucuronic acid, sialic acid, D-galacturonic acid, D-mannuronic acid (*e.g.* polymannuronic acid, or alginic
30 acid), D-glucosamine, D-galactosamine, D-glucose and neuraminic acid including

homopolysaccharides and heteropolysaccharides such as lactose, amylopectin, starch, hydroxyethyl starch, amylose, dextrane sulfate, dextran, dextrans, glycogen, or the polysaccharide subunit of acid mucopolysaccharides, *e.g.* hyaluronic acid; polymers of sugar alcohols such as polysorbitol and polymannitol; heparin or heparon. The polymer
5 prior to cross-linking need not be, but preferably is, water soluble, but the final conjugate must be water soluble. In addition, the polymer should not be highly immunogenic in the conjugate form, nor should it possess viscosity that is incompatible with intravenous infusion or injection if it is intended to be administered by such routes.

Preferably the polymer contains only a single group which is reactive. This helps
10 to avoid cross-linking of protein molecules. However, it is within the scope herein to optimize reaction conditions to reduce cross-linking, or to purify the reaction products through gel filtration or chromatographic sieves to recover substantially homogenous derivatives.

The molecular weight of the polymer may desirably range from about 100 to
15 500,000, and preferably is from about 1,000 to 20,000. The molecular weight chosen will depend upon the nature of the polymer and the degree of substitution. In general, the greater the hydrophilicity of the polymer and the greater the degree of substitution, the lower the molecular weight that can be employed. Optimal molecular weights will be determined by routine experimentation.

The polymer generally is covalently linked to the antagonist though a
20 multifunctional crosslinking agent which reacts with the polymer and one or more amino acid or sugar residues of the antagonist to be linked. However, it is within the scope of the invention to directly crosslink the polymer by reacting a derivatized polymer with the hybrid, or *vice versa*.

Covalent binding to amino groups is accomplished by known chemistries based
25 upon cyanuric chloride, carbonyl diimidazole, aldehyde reactive groups (PEG alkoxide plus diethyl acetal of bromoacetaldehyde; PEG plus DMSO and acetic anhydride, or PEG chloride plus the phenoxide of 4-hydroxybenzaldehyde, succinimidyl active esters, activated dithiocarbonate PEG, 2,4,5-trichlorophenylchloroformate or P-
30 nitrophenylchloroformate activated PEG). Carboxyl groups are derivatized by coupling PEG-amine using carbodiimide. See U.S. 5,739,208; 5,446,090 and 5,672,662.

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Polymers are conjugated to oligosaccharide groups by oxidation using chemicals, *e.g.* metaperiodate, or enzymes, *e.g.* glucose or galactose oxidase, (either of which produces the aldehyde derivative of the carbohydrate), followed by reaction with hydrazide or amino derivatized polymers, in the same fashion as is described by Heitzmann *et al.*, (1974) or Bayer *et al.*, (1979), for the labeling of oligosaccharides with biotin or avidin. Further, other chemical or enzymatic methods which have been used heretofore to link oligosaccharides are particularly advantageous because, in general, there are fewer substitutions than amino acid sites for derivatization, and the oligosaccharide products thus will be more homogenous. The oligosaccharide substituents also are optionally modified by enzyme digestion to remove sugars, *e.g.* by neuraminidase digestion, prior to polymer derivatization.

The polymer will bear a group which is directly reactive with an amino acid side chain, or the N- or C-terminus of the antagonist linked, or which is reactive with the multifunctional cross-linking agent. In general, polymers bearing such reactive groups are known for the preparation of immobilized proteins. In order to use such chemistries here, one should employ a water soluble polymer otherwise derivatized in the same fashion as insoluble polymers heretofore employed for protein immobilization. Cyanogen bromide activation is a particularly useful procedure to employ in crosslinking polysaccharides.

"Water soluble" in reference to the starting polymer means that the polymer or its reactive intermediate used for conjugation is sufficiently water soluble to participate in a derivatization reaction. "Water soluble" in reference to the polymer conjugate means that the conjugate is soluble in physiological fluids such as blood.

The degree of substitution with such a polymer will vary depending upon the number of reactive sites on the antibody, whether all or a fragment of the antibody is used, the molecular weight, hydrophilicity and other characteristics of the polymer, and the particular antibody derivatization sites chosen. In general, the conjugate contains about from 1 to 10 polymer molecules, while any heterologous sequence may be substituted with an essentially unlimited number of polymer molecules so long as the desired activity is not significantly adversely affected. The optimal degree of cross-linking is easily determined by an experimental matrix in which the time, temperature

and other reaction conditions are varied to change the degree of substitution, after which the ability of the conjugates to function in the desired fashion is determined.

The polymer, *e.g.* PEG, is cross-linked by a wide variety of methods known *per se* for the covalent modification of proteins with nonproteinaceous polymers such as PEG.

The long half-life conjugates of this invention are separated from the unreacted starting materials by gel filtration. Heterologous species of the conjugates are purified from one another in the same fashion. The polymer also may be water-insoluble, as a hydrophilic gel.

The thrombolytic compound or agent may be any molecule which breaks up or dissolves a thrombus. Suitable thrombolytic agents include streptokinase, acylated plasminogen-streptokinase activator complex (APSAC), urokinase, single-chain urokinase-plasminogen activator (scu-PA), thrombinlike enzymes from snake venoms such as ancrod (Bell, W. In Colman *et al.*, (1987)), tissue plasminogen activator (t-PA) and biologically active variants of each of the above. The preferred thrombolytic agent is t-PA. Suitable thrombolytic agents which may be used in this invention are disclosed, for example, in U.S. Patent Nos. 5,770,425; 5,770,426; 5,612,029; 5,520,911; 5,736,134; 5,728,567; 5,714,145; 5,840,564; 5,616,486; 5,411,871; 5,520,913; 5,262,170; and 5,108,901.

The thrombolytic agent is administered in a manner similar to the administration of the anti-CD18 antibody described above, and at doses ranging from about 5 to about 20 mg as an intravenous priming dose followed by a continuous intravenous infusion at about 0.1 - 1.0 mg/kg./hr. for 12 hr or about 1 to about 100 mg/dose, preferably from 5 to about 80 mg for the total treatment dose. The thrombolytic agent is generally administered as a IV bolus dose followed by an IV infusion, or as an infusion alone, with the infusion being administered over a time ranging from about 1 hr to about 3 hr. The rate of infusion will generally be about 0.2 - 0.8 mg/kg/hr.

Therapeutic formulations of the anti-CD18 antibody and thrombolytic agent are prepared for storage by mixing these compounds having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (*Remington* (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers,

excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; amino acids such as glycine, glutamine, asparagine, histidine, arginine or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose, trehalose or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or PEG. Alternatively, the compounds of the invention may be separately formulated.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington* (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the anti-CD18 antibody and or thrombolytic agent, which matrices are in the form of shaped articles, *e.g.* films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the Lupron DepotTM (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37 C,

resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by
5 modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release compositions also include liposomally entrapped compounds of the invention. Liposomes containing the compounds of the invention are prepared by
10 methods known in the art, such as described in Epstein *et al.*, (1985); Hwang *et al.*, (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Ordinarily, the liposomes are the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol.% cholesterol, the selected proportion being adjusted for the optimal therapy. Liposomes with enhanced circulation time are disclosed in U.S. Patent No.
15 5,013,556.

The following examples are offered by way of illustration and not by way of limitation. The disclosures of all citations and patents in the specification are expressly incorporated herein by reference in their entirety.

EXAMPLES

Example 1

This study investigated the effect of an anti-CD18 antibody (MHM23) and t-PA in a rabbit model of thromboembolic stroke. In this model, a single blood clot is
25 introduced into the middle cerebral and posterior communicating arteries (which are "main cerebral arteries"). The arterial obstruction (*i.e.*, the clot) remains in place throughout the experiment (unless it is enzymatically removed by t-PA). The following rabbit model is thought to correlate well with the physiological progression of thromboembolic stroke in humans.

The rabbit model of thromboembolic stroke used in this example has been previously described in detail (Bednar *et al.*, (1994); Gross *et al.*, (1993); Kohut *et al.*, (1992); Wilson *et al.*, (1992)). See, also, Gross *et al.*, (1995).

Briefly, New Zealand white rabbits (Charles River, CA) (both males and females; 3.0-3.5kg) were anesthetized with the solution of ketamine (50mg/kg; Aveco Co., Fort Dodge, IA), acepromazine (20 mg, Aveco Co.), and xylazine (5 mg/kg; Mobay Corp., Shawnee, KS), a solution that was subsequently used to maintain sufficient anesthesia for painless surgery (as determined by responses to various physiological and autonomic stimuli, including mean arterial pressure and response to a paw being pinched). After an incision was made in the right femoral triangle to expose the femoral vein and artery, the femoral artery was cannulated with a PE-90 catheter (BD Co., Parsippany, NJ), to which was attached a platinum-iridium electrode. This catheter-electrode permitted the continuous measuring of mean arterial pressure and blood sampling for measurement of arterial blood gases (ABG) (pH, PCO₂, PO₂), hematocrit, and glucose and for determination of hydrogen washout to assess the rCBF by the hydrogen clearance technique (Young, (1980)). After the femoral vein was cannulated with PE-90 tubing for drug infusions, a midline scalp incision was made to expose the calvarium. Bilateral craniectomies were performed and the following were placed; 30-gauge platinum-iridium electrodes to monitor the regional cerebral blood flow (rCBF); a fiberoptic, epidural intracranial pressure (ICP) monitor (Princeton Medical Corp., Hudson, NH); and a temperature sensor (Yellow Springs Instruments, Yellow Springs, OH) to measure brain temperature. All cranial instrumentation was carefully fixed in place with fast-setting epoxy. Through a midline neck incision, the animal was tracheostomized and mechanically ventilated. Both depth and rate of ventilation were modified as needed to maintain ABGs within physiological range.

Throughout the experiment, the brain and core temperatures, mean arterial pressure, and ICP were continuously measured. Additionally, the following parameters were measured before embolization (baseline), at the time of embolization, and hourly after embolization; the rCBF, hematocrit, glucose, and ABG. The mean arterial pressures were kept between 50 and 60 mm Hg throughout the experiment. Fluids (Ringer's lactate or packed cells) were given intravenously as needed (approximately 2-4 ml/kg/h) to

maintain euvoemia. Core and brain temperatures were maintained within 1 C of baseline by using heating blankets and heating lamps.

The autologous clot was prepared by mixing the whole blood (1 ml) with 50 mg of tin granules. The clot was introduced into the PE-90 tubing pretreated with thrombin and was allowed to mature at room temperature.

After tracheostomy, the region of the bifurcation of the common carotid artery was identified, followed by 30 to 60 minutes of equilibration, during which baseline values were obtained. All surgery was completed within 2 hours. Once all the baseline values were obtained, the proximal internal carotid artery and the distal common carotid artery were transiently isolated from the circulation. An arteriotomy was then performed, and the autologous clot embolus was delivered to the anterior circulation of the brain via a catheter advanced into the proximal internal carotid artery. Once embolized, both the proximal internal carotid artery and distal common carotid artery were again isolated from the circulation and an arteriorrhaphy was performed by using 10-0 interrupted nylon sutures. A Philip's dental x-ray machine was used to obtain a submental-vertex radiograph that verified placement of the tin-tagged clot. Embolized clots were noted within the middle cerebral and posterior communicating arteries.

t-PA or a saline solution (0.9% saline) was administered intravenously by continuous infusion from hours 3-5 after the embolization at a total dose of 6.3 mg/kg. MHM23 (2 mg/kg) was administered by bolus dosage 1 hour after embolization. In each instance, the experiment continued for 7 hours after embolization. Submental-vertex radiographs were obtained after embolization and at the end of the experiment. Immediately after the embolization, the rCBF was measured again; the experiment was continued if the rCBF was 15 ml/100 g/ min in any of the three electrodes in the embolized hemisphere (Jones *et al.*, (1981)).

At the end of the experiment, the animals were killed with an overdose of sodium pentobarbital (150 mg/kg), a procedure recognized as acceptable and painless, according to the euthanasia guidelines of the American Veterinary Medical Association. Bilateral thoracotomies were performed in accordance with procedures outlined by the University of Vermont Institutional Animal Care and Utilization Committee. The brain was harvested rapidly and examined grossly for the presence and position of residual clot.

The brain was cut into 2-mm slices in a bread-loaf fashion and incubated in triphenyltetrazolium chloride dye to define the size of the brain infarct (Bose *et al.*, (1988)). This method has been shown to be an acceptable means of determining the size of a brain infarct in our rabbit model and correlates well with hematoxylin and eosin staining (Bednar *et al.*, (1994)). Each brain slice was carefully traced onto clear acetate sheets for later planimetric determination of the infarct size, for which an IBM image analyzer was used. The infarct size was determined according to the modification described by Lin *et al.*, (1993). In this method, the region of the infarct is determined by subtracting the volume of the noninfarcted part of the embolized hemisphere from the entire volume of the nonembolized hemisphere. This modification takes into account that the volume of a brain infarct may be overestimated because of associated swelling.

The analysis of variance for repeated measures was used to analyze the hematocrit, glucose, ABG, rCBF, and ICP in the control and treated groups. If significance was noted, the values of these variables immediately before the t-PA and/or MHM23 administration were then compared by the Student's *t* test. When necessary, the analysis of covariance was used to compare the control and treated groups. After a significant treatment-by-time interaction, individual contrasts were used to compare the treatment means at each time point; that is, if a significant treatment-by-time interaction was noted, the treatment effects were examined at each time point. The infarct size and specific gravities of the brain were compared (treated versus control) by the Student's *t* test. All the results were two-sided and were evaluated by using $p = 0.05$.

The results of the above experiment are depicted in Figs. 1-3. As shown in Figs. 1 and 2, administration of anti-CD18 antibody and t-PA provide an increase in arterial blood flow relative to control. Fig. 3 shows that anti-CD18 antibody and t-PA tend to reduce intracranial pressure (ICP) at 6-7 hours. Furthermore, the experiments show that anti-CD18 antibody is compatible with t-PA and improves the outcome of t-PA.

The increase in cerebral blood flow and the reduction in infarct size observed in the above experiments are thought to be predictive of an improvement in clinical outcome as measured by a standard stroke scale. Accordingly, this application provides a method for improving clinical outcome in patients having suffered stroke as defined herein.

The model described in this example differs from that previously described in Bednar *et al.*, (1992) in that the animals in the study were not subjected to extraneous systemic hypotension (by reducing the mean arterial pressure in the animal to 30 mmHg by controlled exsanguination). Also, the anti-CD18 antibody was given more than 30 minutes after the thromboembolic event and the dose was different.

Example 2

In this study (PDL 2000P), the safety and pharmacokinetics of rhuMAb CD18 (Carter, P. et al., (1992); Eigenbrot, C. et al., (1994)) were studied.

This was a single-blind, placebo-controlled study in healthy subjects using six dose levels (0.06, 0.12, 0.25, 0.5, 1.0, and 2.0 mg/kg/dose) given as two doses 12 hours apart. A total of 37 subjects were recruited. rhuMAb CD18 pharmacokinetics, WBC count, CD 18 binding site saturation (%), antibody formation, and the incidence of adverse events (with particular reference to infection) were evaluated. The results of the PK analyses are summarized in Table 1. The serum concentration profiles are displayed in Fig. 5.

PK analyses could only be completed on subjects who received doses >0.06 mg/kg due to assay sensitivity. Overall, rhuMAb CD18 systemic exposure increased with dose. IV administration of rhuMAb CD18 resulted in serum concentration profiles that were best characterized by a one-compartment model. The volume of distribution (V_d) of 42 to 58 mL/kg approximated the serum volume. Serum clearance (CL) was found to decrease with dose, becoming constant at the two higher dose levels. This was suggestive of nonlinear dose-dependent kinetics with saturation of an elimination process. The terminal half-life ($t_{1/2}$) at doses of 0.5-2.0 mg/kg ranged from 8 to 10 hours. A modest 1.5-fold accumulation was attained, as expected, after administration of the second dose for the 0.5 mg/kg- 2 mg/kg dose groups.

Table 1: Pharmacokinetic Analyses from Study PDL 2000P (mean±SD)

Dose	0.06 mg/kg ^a	0.12 mg/kg	0.25 mg/kg	0.5 mg/kg	1.0 mg/kg	2.0 mg/kg
Parameter	n=3	n=4	n=4	n=4	n=4	n=4
AUC ^b (µg•hr/mL)	ND	12.6±2.0	38.6±7.9	104±22	328±22	485±17
t _{1/2} (hr)	ND	4.1±0.2	6.1±1.2	7.8±1.3	9.5±0.6	9.6±1.1
C _{max} ^c (µg/mL)	ND	2.1±0.4	4.4±0.7	9.2±0.8	24.1±2.0	35.5±5.2
CL (mL/hr/kg)	ND	9.7±1.5	6.7±1.4	4.9±1.0	3.1±0.2	4.1±0.1
V _d (mL/kg)	ND	58.1±11.3	57.6±10.5	54.7±4.5	41.7±3.3	57.2±8.0
C ₁ ^d (µg/mL)	0.8±0.2	2.4±0.6	4.3±1.2	10.1±0.8	25.7±1.6	41.2±7.0
C ₂ ^e (µg/mL)	1.2±0.2	2.8±0.5	6.2±0.5	13.3±0.9	34.7±4.0	52.8±6.6
R ₀ ^f	ND	2.61±0.44	1.55±0.14	1.55±0.17	1.44±0.16	1.34±0.14

Notes: Concentration vs. time data for each subject were fit with a one-compartment model using a weighted least squares non-linear regression estimation procedure.

ND=not determined.

^a PK parameters were not determined for the 0.06 mg/kg dose group due to limit of assay sensitivity.

^b AUC=total area under the serum concentration vs. time curve for a single dose.

^c Maximum predicted serum concentration.

^d Observed maximum concentration after the first dose.

^e Observed maximum concentration after the second dose.

^f Observed accumulation after the second dose.

Example 3

In a second Phase I, single-blind, placebo-controlled study (PDL 2001P), two dose levels of rhuMAb CD18 (1.0 and 2.0 mg/kg) were evaluated in healthy subjects given a single dose (QD) or two doses 12 hours apart (BID). A total of 16 subjects were recruited, all of whom completed the study. Pharmacodynamic measures included the effect of rhuMAb CD18 on the migration of WBCs into skin blisters and saturation of CD18 binding. WBC counts were also evaluated.

Table 2 summarizes the results of the serum PK analyses. Serum concentration profiles are shown in Figs. 6 and 7 for subjects who received single or double doses of rhuMAb CD18, respectively.

5 Table 2: Pharmacokinetic Analyses from Study PDL 2001P (mean±SD)

	1 mg/kg QD n=3	2 mg/kg QD n=3	1 mg/kg BID n=3	2 mg/kg BID n=3
AUC ($\mu\text{g}\cdot\text{hr}/\text{mL}$) ^a	208±46	442±73	233±29	501=47
t _{1/2} (hr)	7.8±1.3	7.0±0.7	9.1±0.2	9.6=1.2
C _{max} ($\mu\text{g}/\text{mL}$) ^b	18.9±4.9	43.5±3.6	17.7±2.0	36.9=7.0
CL (mL/hr/kg)	5.0±1.1	4.6±0.8	4.4±0.6	4.0=0.4
V _d (mL/kg)	55.3±13.4	46.2±3.8	57.0±6.8	55.5=9.5
C ₁ ($\mu\text{g}/\text{mL}$) ^c	18.7±4.9	43.1±3.7	18.7±2.1	39.9=1.1
C ₂ ($\mu\text{g}/\text{mL}$) ^d	NA	NA	24.3±3.7	55.4=11.9
R ₀ ^e	NA	NA	1.54±0.16	1.44±0.08

Notes: Concentration vs. time data for each subject were fit with a one-compartment model using
a weighted least squares non-linear regression estimation procedure. NA=not applicable.

10 ^a Total area under the serum concentration vs. time curve for a single dose.

^b Maximum predicted serum concentration.

^c Observed maximum concentration after the first dose.

^d Observed maximum concentration after the second dose

^e Observed accumulation after the second dose.

15 In general, rhuMAb CD18 pharmacokinetics were consistent with the results from the Phase 1a study. The t_{1/2} was 7-10 hours following single and repeat dose administration. Similarly, the V_d approximated the serum volume. A modest 1.5-fold accumulation was attained, as expected, after administration of the second doses.

20 CD18 binding-site saturation was assessed using FACS analysis. The FACS analysis established that saturation of CD18 receptors on neutrophils was achieved.

An exploratory analysis was performed using a blister technique that had not been validated for assessment of neutrophil migration. This experiment involved the formation of blisters after administration of tetanus or diphtheria toxoids at the blister sites. One group of blisters was formed prior to administration of rhuMAb CD18. The other group of blisters was formed after rhuMAb CD18 administration. Blister fluid was sampled 24 hours after blister formation. WBC counts and differentials were performed on samples of blister fluid. There was no evidence that CD18 inhibited leukocyte margination in this model

Circulating mean WBC counts were raised at all dose levels compared with placebo. The WBC counts fell after 36-48 hours and were at almost baseline levels after 96 hours. Subjects receiving placebo exhibited minimal variation in WBC counts.

Example 4

This experiment studied the extent of interaction of rhuMAb CD18 on the clot lysis activity of the thrombolytic rt-PA. Clot lysis assays were performed in plasma or using purified clot components. There was no apparent in vitro effect of rhuMAb CD18 on rt-tPA clot lysis activity at concentrations of rhuMAb CD18 up to 75 µg/mL.

Example 5

The dosing strategy for a Phase II clinical trial involved a dose that would inhibit neutrophil adhesion without compromising safety, as prolonged inhibition of leukocyte function might result in increased susceptibility to infection (Anderson et al., 1985). The extent of CD18 saturation required to adequately inhibit neutrophil function in preventing reperfusion injury is not precisely known. However, based on the correlation between the extent of CD18 expression and severity of disease in patients with leukocyte adhesion deficiency (LAD), a disease in which mutations in the CD18 gene result in low expression levels, it was postulated that functional inhibition of neutrophil adhesion may require ≥90% saturation of CD18 sites (Anderson et al., 1985). The window for duration of saturation was derived from nonclinical studies in models of AMI that suggested that much of reperfusion injury is completed within 48 hours (Simpson et al., 1988a). Enzymatic estimates of MI size based on plasma levels of creatinine kinase MB bands

(CK-MB) have been shown to correlate well with infarct size as measured at autopsy (Hackel et al., 1984; Grande et al., 1982) and using nuclear scintigraphy (Gibbons, 1991). Based on these serum markers of myocardial injury, the GUSTO trial investigators demonstrated that the majority of myocardial cell death occurs during the first 36 hours following reperfusion therapy (Baardman et al., 1996). Based on these observations, the dosing strategy targeted $\geq 90\%$ saturation of neutrophils for up to 36 hours postdose.

The single, IV bolus administration dose groups of 0.5 mg/kg and 2.0 mg/kg were a result of the integration of PK data from Phase I studies conducted in normal volunteers (PDL 2000P and PDL 2001P), with predicted saturation of CD18 sites derived from the mathematical relationship between rhuMAb CD18 concentration, molecular affinity, and percent receptor occupancy.

Plasma concentrations of rhuMAb CD18 at various times for the 0.5 mg/kg and 2.0 mg/kg dose groups were derived using Phase I PK parameters and one-compartment modeling for IV bolus administration (Model #1, WinNonlin, Scientific Consulting, Inc.) by Equation 1:

$$\text{Equation 1:} \quad C(t) = D/V \cdot e^{(-K_{10} \cdot t)}$$

where:

$c(t)$ = concentration ($\mu\text{g/mL}$) and time (t)

t = time (hours)

D = dose ($\mu\text{g/kg}$)

V = volume (mL/kg)

K_{10} = elimination rate constant (hours^{-1})

Using the concentration data from Equation 1 and an experimentally derived measurement of molecular affinity (K_D) of $0.15 \mu\text{g/mL}$ for the binding of rhuMAb CD18 to the MAC-1 neutrophil receptor in whole, Equation 2 provides a saturation value.

$$\text{Equation 2:} \quad \text{Saturation (S)} = [L] / ([L] + K_D)$$

where:

$[L]$ = concentration of rhuMAb CD18 ($\mu\text{g/mL}$) = $C(t)$

K_D = equilibrium dissociation constant ($\mu\text{g/mL}$)

Using Equations 1 and 2, percent saturation versus time curves can be generated for the 0.5 mg/kg and 2.0 mg/kg doses (see Fig. 4).

Table 3 shows the predicted neutrophil saturations by rhuMAb CD18 and the corresponding times that these saturations were achieved.

5

Table 3: Predicted Neutrophil Saturation

Saturation(%)	-----Dose Group-----	
	<u>0.5 mg/kg</u> Time (hours)	<u>2.0 mg/kg</u> Time/hours
98	3	18
95	12	28
90	20	36
80	30	44
50	44	60
20	60	74

As shown in Table 3, a saturation level of at least 90% is predicted to be maintained for 20 hours in the 0.5 mg/kg dose group and for 36 hours in the 2.0 mg/kg dose group.

10

Randomization occurred after identification of appropriate subjects and procurement of signed informed consent. Site personnel phone into a centralized randomization system (interactive voice response system [IVRS]) for drug kit number assignment. A method of dynamic treatment allocation maximally equalized the numbers of subjects in each treatment group with the same infarct type (anterior vs. other), age, time-to-treatment window (0-6 vs. >6-12 hours), and hospital.

15

rhuMAb CD18 was formulated as a sterile solution aseptically filled in a sterile 10-mL stoppered glass vial. Each vial contains 10 mL of 5.0-mg/mL rhuMAb CD18 aqueous solution (pH 5.0) with 10 mM sodium acetate, 8% trehalose dihydrate, and 0.01% polysorbate 20. No preservatives are used. Placebo equivalents were identical in appearance and excipients to rhuMAb CD18. The manufacturer of rhuMAb CD18 and placebo equivalent was Genentech, Inc., South San Francisco, CA.

20

Following randomization by the IVRS, subjects received a single injection of placebo, 0.5, or 2.0 mg/kg rhuMAb CD18. All doses were administered undiluted. The drug was injected as an IV bolus via syringe into an indwelling catheter. The line was then flushed with 10-mL normal saline solution. All efforts were made to ensure that all
5 subjects receive the study drug prior to or immediately upon initiation of tPa (Activase®) therapy. The total dose of rhuMAb CD18 did not exceed 250 mg.

The accelerated, weight-adjusted GUSTO-I regimen was used for Activase®: 15-mg IV bolus, followed by infusion of 0.75 mg/kg over the first 30 minutes, not to exceed 50 mg; followed by 0.5 mg/kg over the next 60 minutes, not to exceed 35 mg. The total
10 dose of Activase® did not exceed 100 mg. Activase® was stored at a temperature of 2°C-30°C.

Coronary angiography was performed 90 min after the start of Activase® administration to assess TIMI grade flow. The TIMI frame count is the number of cine frames required for opacification of the distal IRA. See Gibson et al., (1996). The count
15 was determined locally (using methods delineated in a TIMI frame-count methods video) and by the Core Angiography reader. The TIMI flow grading is classified as follows:

- Grade 0: No perfusion
- Grade 1: Penetration without perfusion
- Grade 2: Partial penetration
- 20 Grade 3: Complete perfusion.

This study shows that treatment with both a thrombolytic compound (tPA) and an anti-CD18 antibody provides a statistically significant improvement in the TIMI grade blood flow in patients relative to patients treated with the thrombolytic compound alone, for example, the percentage of patients with TIMI grade 3 flow in the IRA 90 min after
25 the start of thrombolytic therapy is increased.

While the invention has necessarily been described in conjunction with preferred embodiments and specific working examples, one of ordinary skill, after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and alterations to the subject matter set forth herein, without departing from
30 the spirit and scope thereof. Hence, the invention can be practiced in ways other than

those specifically described herein. It is therefore intended that the protection granted by letters patent hereon be limited only by the appended claims and equivalents thereof.

The entire contents and disclosure of parent application USSN 08/788,800 as well as all other references and patents disclosed are incorporated herein by reference.

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